THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 259, No. 4, Issue of February 25, pp. 2227-2235, 1984
Printed in U.S.A.

Agonist-induced Changes in β-Adrenergic Receptors on Intact Cells*

(Received for publication, July 20, 1983)

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Competition by β-adrenergic agonists and antagonists for ^{125}I-pindolol binding sites on intact cells (1321N1 human astrocytoma and C62B rat glioma) was measured using short time binding assays as previously described (Toews, M. L., Harden, T. K., and Perkins, J. P. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 3553–3557). Preincubation of cells with agonists converted about half of the cellular β-adrenergic receptors from a form exhibiting high affinity for the agonists isoproterenol and epinephrine and the antagonist sotalol to a form exhibiting much lower *apparent* affinity for these ligands in short time assays. Exposure to agonists did not alter the affinity of receptors for the antagonist metoprolol. This change in the ligand binding properties of the receptor was rapid ($t_{1/2}$ = 1–2 min following a lag of about 0.5 min), reversible ($t_{1/2}$ = 6–8 min), and dependent on the agonist concentration present during the preincubation ($K_{0.5}$ = 15 nM for isoproterenol). Both isoproterenol and sotalol attained equilibrium with the high affinity receptors very rapidly but equilibrated only slowly with those receptors exhibiting low apparent affinity in short time assays. These results are interpreted in terms of a model which postulates that both the low apparent affinity in short time assays and the subsequent slow equilibration of hydrophilic ligands with these receptors result from agonist-induced internalization of a fraction of cell surface β-adrenergic receptors. The relationship of this change in receptor binding properties to other aspects of agonist-induced desensitization of the β-adrenergic receptor-coupled adenylate cyclase system is discussed.

The phenomenon of desensitization of receptor-mediated hormone responses upon prolonged incubation in the presence of hormone is well documented (2–4). In the case of β-adrenergic receptors, incubation of cells in the presence of agonists has been shown to lead to dramatic changes in both the physical and pharmacological properties of these receptors measured subsequently in membrane preparations (2–4). Recently developed methodology has made it feasible to study β-adrenergic receptors on intact cells as well as in membrane preparations (5–8). We have been studying ^{125}I-PIN[1] binding to β-adrenergic receptors on intact cells (1) as part of a continuing investigation of the changes that occur in β-adrenergic receptors during the process of agonist-induced desensitization.

In a previous manuscript (1) we measured binding of ^{125}I-PIN to intact cells in short time assays under conditions approximating the initial velocity of binding of the radioligand. The results clearly demonstrated that in both 1321N1 human astrocytoma cells and C62B rat glioma cells the native state of the β-adrenergic receptor was predominantly one with high affinity for agonists. A small fraction of ^{125}I-PIN binding sites exhibited very low apparent affinity[2] for agonists under these conditions. As in previous studies (5–8), only low affinity binding was observed in longer time equilibrium binding assays. In contrast, the observed affinity of intact cell receptors for antagonists was the same when measured in equilibrium assays as when measured in short time assays. These results, like those of Pittman and Molinoff (8), suggested that an agonist-induced change in agonist binding properties of the receptor was occurring in these cells. In the experiments reported here we have further used the short time assay approach to investigate changes in the ligand binding properties of intact cell β-adrenergic receptors that occur during incubation in the presence of agonists. A discussion of the possible relationship of these changes to the various other aspects of the phenomenon of desensitization also is presented.

EXPERIMENTAL PROCEDURES

Materials—(−)-Isoproterenol bitartrate, (−)-epinephrine bitartrate, and (±)-propranolol hydrochloride were obtained from Sigma. [^{3}H]Adenine and [^{125}I]sodium iodide were obtained from ICN and Amersham Corp., respectively. The following drugs were generous gifts: (−)-pindolol from Sandoz Pharmaceuticals, (±)-sotalol hydrochloride and soterenol hydrochloride from Bristol-Myers Co., and (±)-metoprolol and terbutaline sulfate from Drs. P. B. Molinoff and B. B. Wolfe (Department of Pharmacology, University of Pennsylvania, Philadelphia, PA), respectively. ^{125}I-PIN was prepared by a modification of the method of Barovsky and Brooker (7).

Cell Culture—Human astrocytoma cells (1321N1) and rat glioma cells (C62B, passage 30–40) were grown as previously described (1). Cells taken from confluent flasks were seeded in 35-mm culture dishes (Falcon) at a density of 80,000 cells/dish and used on the 4th day following subculture.

Intact Cell Receptor Binding Assays—Intact cells were incubated at 37 °C with various concentrations of β-adrenergic ligands in growth

* This investigation was supported in part by Research Grant GM29522. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Individual Postdoctoral Fellowship Grant GM-08837 from the United States Public Health Service.

[1] The abbreviations used are: ^{125}I-PIN, ^{125}I-(−)-pindolol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

[2] In many of the experiments reported here, the competing ligands do not attain equilibrium with a subpopulation of cellular β-adrenergic receptors during the short time assays employed to study their interaction with the receptors. The IC_{50} values obtained therefore are not reflective of the true affinity of the receptor for these ligands but rather represent an "apparent affinity" for these ligands under the particular assay conditions employed. While the model used for computerized curve-fitting is not entirely correct in these instances, the shape of a "pre-equilibrium" competition curve is sufficiently similar to that for the equilibrium situation to allow use of this model for estimating the fractions of receptors in the high and low apparent affinity forms.

medium containing 1 mM sodium ascorbate. Control cells were incubated under the same conditions with ascorbate only. The cell sheets were then rinsed 2 or 3 times with serum-free Eagle's medium containing 20 mM Hepes (pH 7.4) at 37 °C. Binding to intact cell β-adrenergic receptors in the presence of varying concentrations of competing ligands was then measured in 15-s assays with 100 pM ^{125}I-PIN as previously described (1). In some experiments, desensitized cells were incubated for different times with varying concentrations of competing ligand to allow equilibration of the ligand with the various forms of the receptor prior to performing short time binding assays. Specific binding in the absence of competing drugs in 1321N1 cells was approximately 1500 cpm bound/assay and in C62B cells was approximately 500 cpm bound/assay. Nonspecific binding, measured in each experiment as that occurring in the presence of 1 μM propranolol, was about 15% of total binding for 1321N1 cells and 30% for C62B cells. In all cases where tested, the results obtained with control cells (incubated with ascorbate) were essentially identical with those obtained with naive cells (1).

Cyclic AMP Accumulation in Intact Cells—Intact cells were incubated at 37 °C in the absence or presence of isoproterenol in growth medium containing 1 mM sodium ascorbate. The cell sheets were then rinsed three times (2 ml each) with Eagle's-Hepes at 37 °C. Cellular accumulations of cyclic AMP in response to isoproterenol were then measured using a modification of the method of Shimizu *et al.* (9) similar to that previously described (10). One ml of Eagle's-Hepes containing 1 mM sodium ascorbate, [^{3}H]adenine (approximately 5 μCi/ml), and varying concentrations of isoproterenol was added to each dish. Following a 1-min incubation at 37 °C this medium was aspirated and 1 ml of 5% trichloroacetic acid was added. The amounts of [^{3}H]ATP and [^{3}H]cAMP present in the trichloroacetic acid-soluble fractions were then determined using a modification (11) of the method of Salomon *et al.* (12).

Data Analysis—As documented in a previous report (1), the IC_{50} obtained for a competing ligand in these short time assays under conditions approximating the initial rate of radioligand binding provides an estimate of the true affinity of the receptor for competing ligands that attain equilibrium with the receptor very rapidly. For competing ligands that attain equilibrium slowly relative to the time of the assay, the observed IC_{50} will be larger than the true K_D, and changes in the observed IC_{50} with time can provide a measure of the approach to equilibrium of the competitor with the receptor (13, 14).

Competition curves were analyzed by computerized nonlinear least squares curve fitting of the raw data using the Gauss-Newton or Marquardt methods (15). The model used was that for law of mass action interaction of the competing ligand with either a single site ($n = 1$) or two independent sites ($n = 2$) as follows:

$$B_s = \sum_{i=1}^{n} B_i - \sum_{i=1}^{n} \frac{B_i S}{I_i + S} + N$$

where s is the concentration of competing ligand, B_s is the concentration of ^{125}I-PIN bound at a given value of S, B_i is the concentration of ^{125}I-PIN bound to each site in the absence of competitor, I_i is the concentration of competing ligand which reduces the amount of ^{125}I-PIN bound to site i by 50% (the IC_{50}), and N is the concentration of nonspecific binding sites. It should be noted that this model will be correct only if the competing ligand is at equilibrium with all specific binding sites under the conditions of the assay.[2]

Comparison of the single site and two-site models was done as previously desribed (16). Values reported for fractions of high and low apparent affinity binding sites are reported as arithmetic means; IC_{50} and K_D values are reported as geometric means (17). For all competition curves, data are expressed as the percentage of ^{125}I-PIN specifically bound in the absence of competitor. The averages of the experimental data points are shown together with the curve for the computer-derived fit of the data. The amount of binding in the presence of 1 μM propranolol (nonspecific) is also indicated. Standard errors of all points on the curves were generally less than 5% of the amount specifically bound in the absence of competitor.

RESULTS

Agonist-induced Conversion of Intact 1321N1 Cell β-Adrenergic Receptors to a Form with Low Apparent Affinity[2] For Isoproterenol in Short Time Assays—Human astrocytoma cells (1321N1) were incubated for 20 min at 37 °C in the absence or presence of various concentrations of isoproterenol. The cell sheets were then rinsed two times over a period of 1 min to remove the preincubation medium, in particular to reduce the isoproterenol concentration by several orders of magnitude. Isoproterenol competition for ^{125}I-PIN binding was then measured in 15-s binding assays (Fig. 1). In all cases the data were significantly better fit ($p < 0.05$) by a two-site law of mass action binding model than by a single-site model. In control cells the majority of ^{125}I-PIN binding (83 ± 5%, $n = 3$) was inhibited with an IC_{50} of 53 ± 17 nM, whereas the remainder (17%) was inhibited only at much higher concentrations of isoproterenol (IC_{50} = 520 ± 930 μM). With increasing concentrations of isoproterenol during the preincubation period, there was a progressive decrease in the fraction of receptors exhibiting high affinity for isoproterenol and a corresponding increase in the fraction of receptors exhibiting lower apparent affinity for isoproterenol. The amount of ^{125}I-PIN bound in the absence of competitor remained constant or decreased slightly.[3] Changes in the IC_{50} values for isoproterenol at the two types of ^{125}I-PIN binding sites were very small. Thus, following preincubation with 10 nM isoproterenol, 61 ± 5% ($n = 2$) of ^{125}I-PIN binding was inhibited with an IC_{50} of 170 ± 82 nM and the remaining 39% was inhibited with an IC_{50} of 710 ± 600 μM. When the preincubation was with 1 μM isoproterenol, 37 ± 6% ($n = 3$) of ^{125}I-PIN binding was inhibited with an IC_{50} of 120 ± 90 nM and the remaining

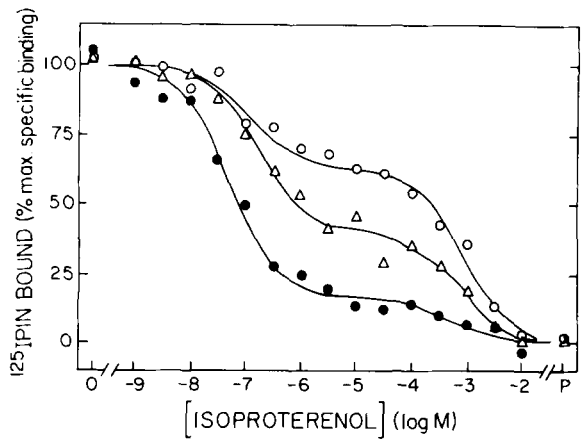

FIG. 1. **Isoproterenol competition curves with 1321N1 cells preincubated without or with isoproterenol.** 1321N1 cells were incubated at 37 °C for 20 min without (●) or with 10 nM (△) or 1 μM (○) isoproterenol and then rinsed twice. Binding of ^{125}I-PIN to intact cells was then measured in 15-s assays in the presence of the indicated concentrations of isoproterenol as described under "Experimental Procedures." The data points are the average of four determinations from two or three separate experiments.

[3] In the experiments reported here, the amount of ^{125}I-PIN bound in the absence of competitor to cells preincubated with 1 μM isoproterenol was 95 ± 10% ($n = 6$) of the amount bound to control cells under the same conditions. In early experiments (not shown) cells were rinsed only once following preincubation with isoproterenol and then 1-min binding assays were performed using 25 pM ^{125}I-PIN. In these assays, desensitized cells exhibited 10–25% more binding in the absence of competitor than did control cells. This increase in the rate of radioligand binding to desensitized cells occurred in spite of a 10–15% decrease in the total number of assayable receptors as measured in membrane preparations from such cells or in intact cells assayed for 1 min with extremely high concentrations of radioligand so that all receptors would be labeled even in a 1-min assay. Whether the failure to observe this phenomenon in the assays reported here results from the more effective removal of isoproterenol with additional rinses or from the shorter assay time and higher concentration of radioligand employed has not been determined. This result may be related to a similar change in the apparent rate of antagonist binding to S49 lymphoma cells reported by Reynolds *et al.* (18).

63% was inhibited with an IC_{50} of 840 ± 41 μM.

Slow Equilibration of Isoproterenol with Those Receptors Exhibiting Low Apparent Affinity in Short Time Assays—The results above show that incubation of cells in the presence of a β-adrenergic agonist converts a portion of the β-adrenergic receptors from a form exhibiting high affinity for isoproterenol to a form of lower apparent affinity. However, the IC_{50} value for this form of the receptor observed following preincubation with agonist was considerably higher than the K_D value of 11 μM obtained in equilibrium competition binding assays with isoproterenol (1). If isoproterenol came to equilibrium slowly with this altered form of the receptor present in cells preincubated with isoproterenol, then competition curves obtained at short times of binding might be expected to be shifted to the right relative to the curve obtained at equilibrium (1). The experiment shown in Fig. 2 demonstrates that in fact this was the case. Following 20 min preincubation with isoproterenol (1 μM) to induce formation of the altered form of the receptor and washing to remove isoproterenol, cells were further incubated with the indicated concentrations of isoproterenol in the absence of ^{125}I-PIN for various times to allow isoproterenol to approach equilibrium with the altered form of the receptor. Binding of ^{125}I-PIN to these cells was then determined in 15-s assays in the continued presence of the indicated concentrations of isoproterenol. The curve obtained without pre-equilibration with isoproterenol (from Fig. 1) is included for comparison. With increasing time of equilibration with isoproterenol, there was a progressive leftward shift of the "low affinity portion" of the competition curves. This is the result expected if isoproterenol comes to equilibrium slowly with the altered form of the receptor. Following a 1-min equilibration with isoproterenol, 38 ± 7% (n = 3) of ^{125}I-PIN binding was inhibited with an IC_{50} of 66 ± 52 nM and the remaining 62% was inhibited with an IC_{50} of 69 ± 34 μM. With 2- or 5-min equilibration (data not shown) there was a further leftward shift in this portion of the competition curves. There was no change in the high affinity portion of the competition curves, indicating that isoproterenol equilibrates very rapidly with the high affinity receptors remaining in these cells just as in control cells.

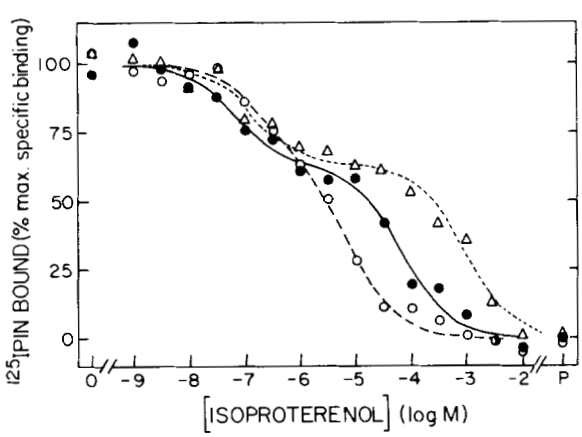

FIG. 2. **Apparent slow equilibration of isoproterenol with low affinity receptors.** 1321N1 cells were incubated at 37 °C for 20 min in the presence of 1 μM isoproterenol and then rinsed three times. One ml of Eagle's-Hepes containing 1 mM sodium ascorbate and the indicated concentrations of isoproterenol was then added to each dish and the cells were further incubated at 37 °C for either 1 min (●) or 60 min (○). This medium was then aspirated and ^{125}I-PIN binding was measured in 15-s assays as described under "Experimental Procedures." The curve from Fig. 1 for cells with no "pre-equilibration" with isoproterenol between the "preincubation" and assay steps is included for comparison (△). The data are the averages of three experiments with duplicate determinations at each point.

Following 60 min equilibration, 34 ± 16% (n = 3) of ^{125}I-PIN binding appeared to be inhibited with an IC_{50} of 180 ± 220 nM and the remaining 66% with an IC_{50} of 7.9 ± 4.9 μM. This latter value is essentially the same as that obtained previously in equilibrium competition binding assays with naive cells (11 μM, Ref. 1) and is about 100-fold higher than the high affinity value obtained with control cells (Fig. 1). However, it should be noted that this curve may reflect processes in addition to competition by isoproterenol for ^{125}I-PIN binding sites. At the very low concentrations of isoproterenol, some reversal of the agonist-induced change in binding properties of the receptors is most likely occurring during the 60-min incubation, whereas at the higher concentrations of isoproterenol, agonist-induced loss of receptors will be occurring (19). Thus, both the total number of receptors present and their distribution among the various forms being studied will probably be different for each point on this portion of the competition curve. We believe it is most likely that the apparent high affinity competition observed under these conditions actually represents a dose-response curve for agonist-induced receptor loss.

Antagonist Binding Properties of Receptors on Agonist-pretreated 1321N1 Cells—The results presented above show that preincubation of cells with isoproterenol leads to a dramatic change in the agonist binding properties of a portion of the cellular β-adrenergic receptors. To determine whether the antagonist binding properties of intact cell receptors were also changed following exposure to isoproterenol, short time competition binding assays were performed with the antagonists metoprolol and sotalol on cells preincubated without or with 1 μM isoproterenol. Preincubation with isoproterenol did not cause a significant change in the shape or position of the competition curve obtained with metoprolol (Fig. 3*A*). In both cases the curves indicated interaction of metoprolol with a single class of sites, with an IC_{50} of 390 ± 98 nM (n = 2) in control cells and 500 ± 210 nM (n = 2) in cells preincubated with isoproterenol.

The curves obtained with the antagonist sotalol (Fig. 3*B*) were better fit by a two-site binding model than by a single-site model and in fact were quite similar to those obtained with isoproterenol (Fig. 1). With control cells, 85 ± 5% (n = 3) of ^{125}I-PIN binding was inhibited with an IC_{50} of 210 ± 72 nM and the remaining 15% was inhibited with an IC_{50} of 320 ± 670 μM. In cells preincubated with isoproterenol, 29 ± 8% (n = 3) of ^{125}I-PIN binding was inhibited with an IC_{50} of 200 ± 250 nM and the remaining 71% was inhibited with an IC_{50} of 180 ± 100 μM.

Competition by sotalol for ^{125}I-PIN binding sites also was measured in 15-s assays with naive cells and with cells preincubated for 60 min with varying concentrations of sotalol prior to assay (Fig. 4). The curve obtained with naive cells was similar to that shown for control cells in Fig. 3, with 79 ± 5% (n = 2) of ^{125}I-PIN binding inhibited with an IC_{50} of 100 ± 30 nM and the remaining 21% inhibited with an IC_{50} of 240 ± 270 μM. The data obtained with cells pre-equilibrated with sotalol prior to assay indicated that nearly all (93 ± 3%, n = 4) of the ^{125}I-PIN binding sites under these conditions exhibited high affinity for sotalol (IC_{50} = 77 ± 10 nM) and that very few receptors remained in the low apparent affinity form (7%, IC_{50} = 960 ± 2000 μM). These results suggest that the binding sites exhibiting low apparent affinity for sotalol in 15-s assays in fact result from slow equilibration of sotalol with a population of receptors which exhibit high affinity once equilibrium is attained.

Time and Concentration Dependence of the Change in Re-

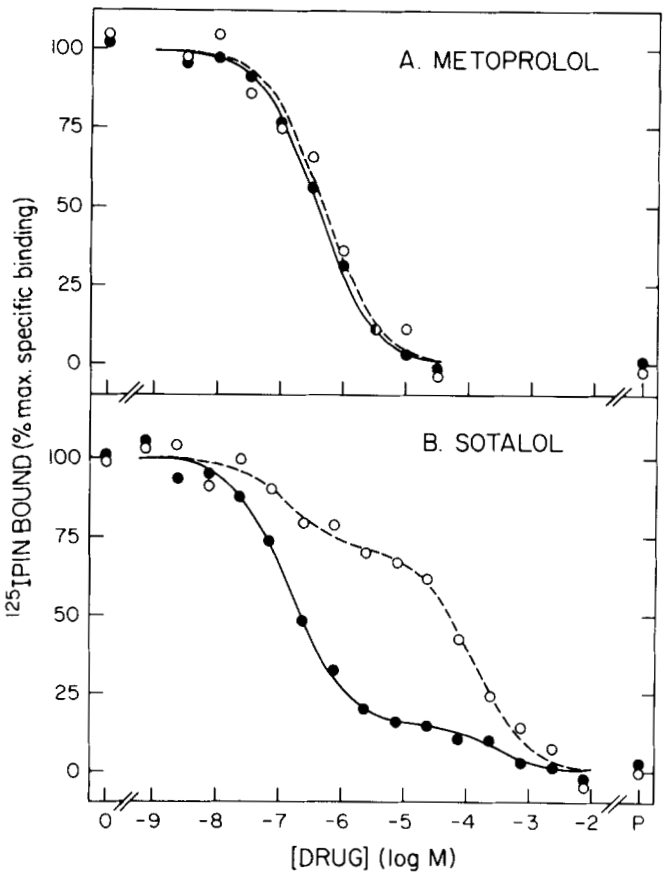

FIG. 3. **Antagonist competition curves from cells preincubated without and with isoproterenol.** 1321N1 cells were preincubated without (●) or with (○) 1 μM isoproterenol at 37 °C for 20 min and then rinsed twice prior to performing 15-s binding assays in the presence of the indicated concentrations of metoprolol (*A*) or sotalol (*B*). The data are the averages of two or three experiments for each curve.

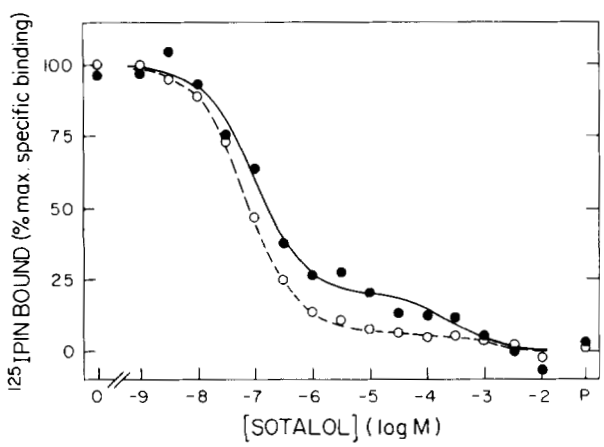

FIG. 4. **Sotalol competition curves with naive 1321N1 cells.** 1321N1 cells were rinsed once with Eagle's-Hepes and then either assayed immediately in 15-s assays in the presence of the indicated concentrations of sotalol (●) or incubated for 60 min in the presence of the indicated concentrations of sotalol prior to 15-s assays in the presence of the indicated concentrations of sotalol (○). The data points are the averages of 2–4 separate experiments.

ceptor Binding Properties and Its Reversal—To further study the preincubation time and concentration dependence of this change in receptor binding properties and its reversal, the ability of 10 μM isoproterenol to inhibit specific binding of ^{125}I-PIN was measured in 15-s assays following preincubation under various conditions. From the data in Fig. 1 it can be seen that the per cent of specific binding in the presence of 10 μM isoproterenol provides a good estimate of the fraction of receptors exhibiting altered binding properties. The time course for formation of this form of the receptor during incubation with 1 μM isoproterenol is shown in Fig. 5*A*. There was a lag of 30–45 s before any change in binding was detected; the reaction then occurred rapidly, with a $t_{1/2}$ of 1–2 min, and

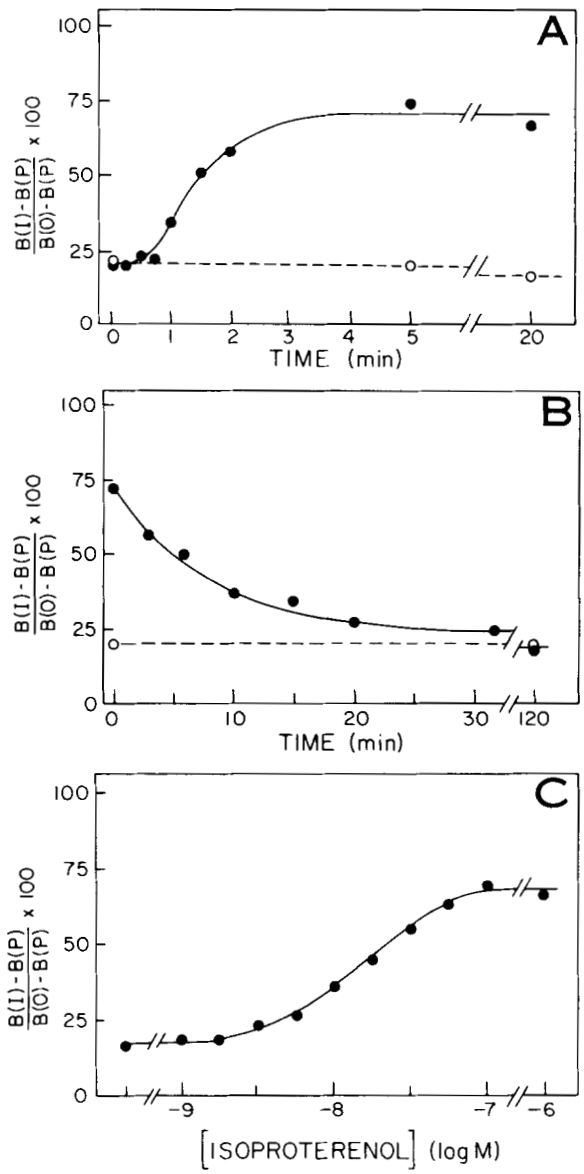

FIG. 5. **Time and isoproterenol concentration dependence of formation of the low affinity form of intact cell receptors.** In each of the experiments presented above, 1321N1 cells were incubated at 37 °C without (○) or with (●) isoproterenol as described below and then rinsed three times with Eagle's-Hepes at 37 °C. ^{125}I-PIN binding was then measured in 15-s assays in the absence of competitor (*B*(*O*)), in the presence of 10 μM isoproterenol (*B*(*I*)), or in the presence of 1 μM propranolol (*B*(*P*), nonspecific binding). The specific binding in the presence of 10 μM isoproterenol (*B*(*I*)-*B*(*P*)) is expressed as a percentage of specific binding in the absence of competitor (*B*(*O*)-*B*(*P*)). *A*, cells were incubated with 1 μM isoproterenol for the times indicated prior to performing binding assays. *B*, cells were incubated with 1 μM isoproterenol for 20 min, washed 3 times with growth medium, and then further incubated at 37 °C for the indicated times prior to performing binding assays. *C*, cells were incubated for 20 min in the presence of the indicated concentrations of isoproterenol prior to performing binding assays. The data points are the averages of two experiments.

appeared to have reached a steady state level by 5 min, with 60–70% of the receptors exhibiting low apparent affinity. With longer times of incubation (up to 2 h, data not shown), the total number of ^{125}I-PIN binding sites began to decrease, but the fraction of these sites exhibiting high and low apparent affinity for isoproterenol remained constant. The reversal reaction was monitored following 20 min incubation with 1 μM isoproterenol and then three washes to remove isoproterenol. The reversal reaction occurred with a $t_{1/2}$ of 6–8 min and was complete by 60 min (Fig. 5*B*). Thus, the forward reaction is slow enough that conversion of receptors to the altered form during the 15-s assays should not contribute to the results obtained in the various experiments using 15-s assays. Likewise, the reversal reaction is slow enough that no significant reversal should be occurring during the 1 min of washing employed between the preincubation and assay steps. The extent of conversion of receptors to the form exhibiting low apparent affinity for isoproterenol also was measured following 20 min preincubation with various concentrations of isoproterenol (Fig. 5*C*); half-maximal conversion of receptors to this form was observed with 15 nM isoproterenol.

Ligand Specificity for Formation and Detection of the Altered Form of Intact Cell Receptors—Assays similar to that above were used to investigate the ability of a variety of ligands to induce conversion of receptors to the form exhibiting low apparent affinity for isoproterenol (Table I). All agonists tested induced similar changes in the binding properties of the receptor for the agonists isoproterenol and epinephrine and the antagonist sotalol. The partial agonists soterenol and terbutaline induced smaller conversions of receptors to the altered form than did the full agonists. Furthermore, their efficacies (relative to isoproterenol) for inducing this change in the binding properties of the receptor were similar to their relative efficacies for stimulation of cyclic AMP accumulation in intact 1321N1 cells (about 35% for soterenol and 60% for terbutaline). Antagonists had no effect on the distribution of receptors between the two forms. None of the ligands tested altered the apparent affinity of the receptor for metoprolol.

Isoproterenol-stimulated Cyclic AMP Accumulation in Control and Desensitized 1321N1 Cells—The possible relationship between the conversion of receptors to the low apparent affinity form and the loss of responsiveness to isoproterenol of the cyclic AMP generating system of 1321N1 cells was investigated. Cells were preincubated with varying concentrations of isoproterenol for 20 min, washed, and then cyclic AMP accumulation was measured in response to varying concentrations of isoproterenol in 1-min assays (Fig. 6). With increasing concentrations of isoproterenol during the preincubation, a progressive decrease in the maximal level of cyclic AMP accumulation was observed. A small increase in the value of K_{act} from 15 nM in control cells to 50 nM in cells preincubated with isoproterenol also was observed. These K_{act} values are similar to the K_D values for binding of isoproterenol to the high affinity form of the receptor on intact cells. It should be noted that maximal cyclic AMP accumulation occurred by 1 μM isoproterenol and that no further increase in cyclic AMP accumulation was observed in either control or desensitized cells at the higher concentrations of isoproterenol where binding to the low apparent affinity form of the receptor occurs. Thus, the receptors exhibiting the low apparent affinity for isoproterenol in short time assays appear to be uncoupled from the cyclic AMP generating system. Furthermore, the fractional stimulation of cAMP accumulation remaining following various preincubation conditions correlates well with the fraction of receptors remaining in the high agonist-affinity form (Fig. 1).

Intact Cell Binding Studies with Control and Desensitized C62B Cells—To determine whether similar changes in receptor binding properties occur upon exposure of C62B cells to agonists, analogous experiments were performed with these cells. Fig. 7 shows the competition curves obtained in 15-s assays with isoproterenol, metoprolol, and sotalol in C62B cells preincubated without or with isoproterenol (1 μM for 20 min). In all cases, preincubation with isoproterenol led to a 20–30% decrease in the amount of ^{125}I-PIN bound in the absence of competing ligand. The competition curve obtained with isoproterenol (Fig. 7*A*) in control cells, like that previously obtained in naive cells (1), was better fit by a two-site binding model, with 69 ± 5% (n = 3) of ^{125}I-PIN binding inhibited with an IC_{50} of 83 ± 28 nM and the remaining 31%

TABLE I

Ability of β-adrenergic ligands to induce and detect a low apparent affinity form of the β-adrenergic receptor

1321N1 cells were incubated for 20 min at 37 °C in the presence of the indicated concentrations of β-adrenergic receptor ligands. The cells were then washed 2–3 times with Eagle's-Hepes at 37 °C. ^{125}I-PIN binding was then measured in 15-s assays at 37 °C in the presence of the indicated concentrations of various competing ligands. Binding in the presence of the competing ligands is expressed as a percentage of the total specific binding in the absence of competitor. The results shown are the averages of 2–3 separate experiments; standard errors were generally less than 5% of the total specific binding in the absence of competitor.

Preincubation	% binding in presence of			
	Isoproterenol (10 μM)	Epinephrine (10 μM)	Sotalol (10 μM)	Metoprolol (0.3 μM)
Control	12	23	16	54
1 μM Isoproterenol	71	68	58	50
1 μM Epinephrine	78	79	74	48
1 μM Sotalol[a]	4	14	2	53
1 μM Metoprolol	14	21	16	54
1 μM Soterenol[b]	26			
10 μM Terbutaline[b]	40			

[a] The decrease in binding to the low apparent affinity form of the receptor observed following preincubation with sotalol may reflect incomplete dissociation and removal of sotalol during the 1-min wash prior to the binding assay, due to its relatively high affinity and slow membrane permeability.

[b] No greater formation of the altered form of the receptors was observed when the preincubation was done with 10-fold higher concentrations of these partial agonists, indicating that the concentrations used were maximally effective.

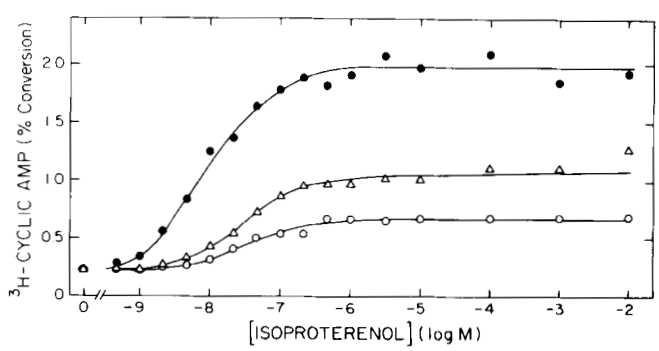

FIG. 6. **Accumulation of cyclic AMP in response to isoproterenol by 1321N1 cells.** Cells were incubated at 37 °C for 20 min without (●) or with 10 nM (△) or 1 μM (○) isoproterenol and then rinsed three times with Eagle's-Hepes. The amount of [^{3}H]cAMP formed in response to the indicated concentrations of isoproterenol was then measured as described under "Experimental Procedures." The data points shown are the averages of duplicate determinations from a single experiment. Similar results were obtained in two additional experiments.

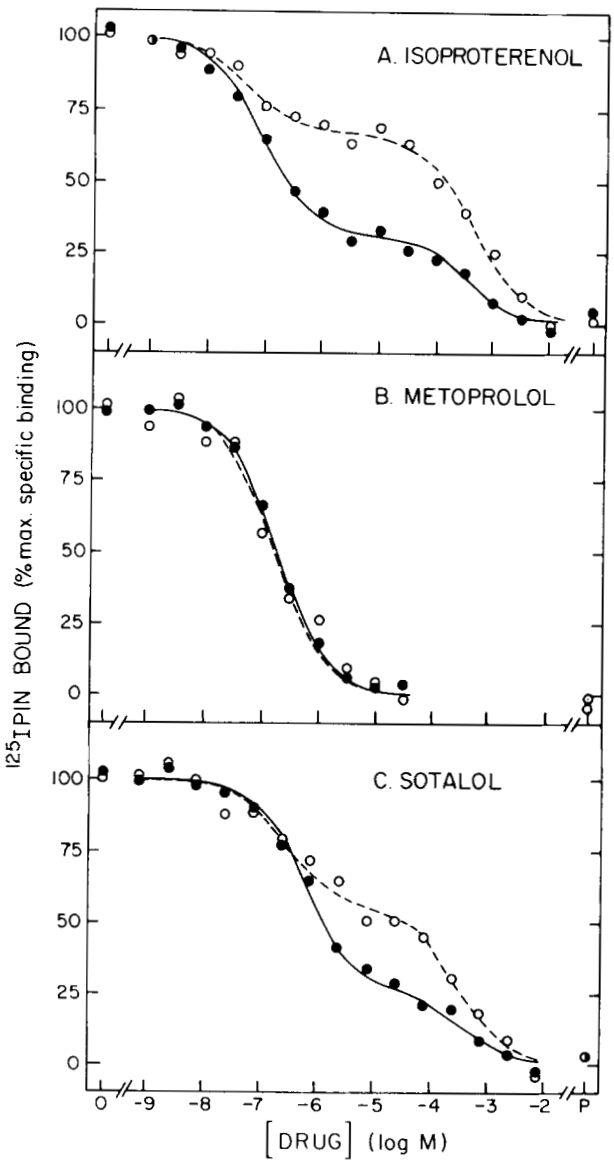

FIG. 7. **Competition curves obtained with C62B cells incubated without or with isoproterenol.** C62B cells were incubated for 20 min at 37 °C without (●) or with (○) 1 μM isoproterenol and then rinsed three times. Binding of ^{125}I-PIN was then measured in 15-s assays in the presence of the indicated concentrations of isoproterenol, metoprolol, or sotalol. The data are the averages of 2–4 separate experiments for each curve.

inhibited with an IC_{50} of 320 ± 260 μM. In cells preincubated with isoproterenol (Fig. 7*A*), 34 ± 5% (n = 3) of ^{125}I-PIN binding was inhibited with an IC_{50} of 54 ± 45 nM and the remaining 66% was inhibited with an IC_{50} of 470 ± 220 μM. The curves obtained with metoprolol (Fig. 7*B*) in both control cells and cells preincubated with isoproterenol indicated interaction with a single class of sites; in control cells the IC_{50} was 200 ± 46 μM (n = 2), and in cells preincubated with isoproterenol the IC_{50} was 170 ± 58 μM (n = 2). The competition curves obtained with sotalol (Fig. 7*C*), like those for isoproterenol, indicated interaction with two sites. In control cells, 74 ± 5% (n = 3) of ^{125}I-PIN binding was inhibited with an IC_{50} of 870 ± 260 nM and the remaining 26% was inhibited with an IC_{50} of 550 ± 540 μM. In cells preincubated with isoproterenol, 44 ± 6% (n = 3) of ^{125}I-PIN binding was inhibited with an IC_{50} of 350 ± 220 nM and the remaining 56% was inhibited with an IC_{50} of 400 ± 220 μM. Thus, the

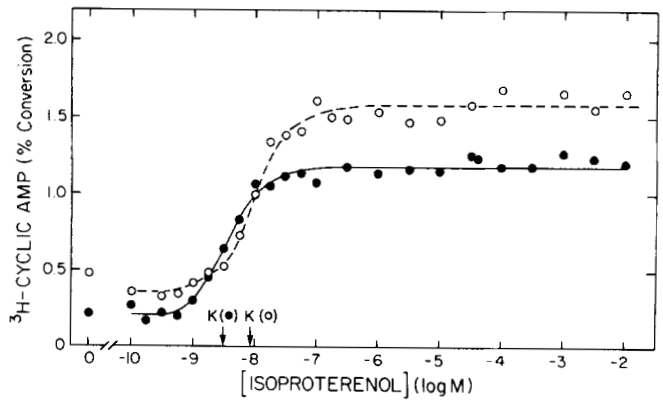

FIG. 8. **Accumulation of cyclic AMP in response to isoproterenol by C62B cells.** C62B cells were incubated without (●) or with (○) 1 μM isoproterenol for 20 min and then rinsed three times with Eagle's-Hepes. The amount of [^{3}H]cAMP formed in response to the indicated concentrations of isoproterenol was then measured as described under "Experimental Procedures." The data points shown are from a single experiment with duplicate determinations at each point. Similar results were obtained in two additional experiments.

results obtained with all three ligands in C62B cells are analogous to those obtained with the 1321N1 cells.

Isoproterenol-stimulated Cyclic AMP Accumulation in Control and Desensitized C62B Cells—The effect of preincubation with isoproterenol on subsequent isoproterenol-stimulated cyclic AMP accumulation in 1-min assays also was measured in C62B cells (Fig. 8). Following 20 min preincubation with 1 μM isoproterenol, there was an increase in K_{act} from 2–3 to 8–10 nM and a 30–40% increase in the maximal response observed. The apparent increase in the basal level of cyclic AMP accumulation in cells preincubated with isoproterenol most likely results from incomplete removal of isoproterenol prior to the assay; however, residual isoproterenol from the preincubation step cannot explain the increase in maximal response observed in the presence of higher concentrations of isoproterenol. With longer times of incubation (data not shown) there was further loss of ^{125}I-PIN binding sites and a progressive decrease in maximal response; however, a portion of this decrease may be due to heterologous types of desensitization (20) rather than simply due to further decreases in the number of high affinity receptors.

DISCUSSION

In short time assays with control 1321N1 cells, isoproterenol distinguishes two populations of receptors, the majority (about 85%) exhibiting high affinity for isoproterenol (50–100 nM) and the remainder exhibiting very low apparent affinity[2] (500–1000 μM). Exposure of these cells to isoproterenol for a period prior to such short time assays does not change the total amount of ^{125}I-PIN binding in the absence of competitor, but rather causes a decrease in the fraction of high affinity receptors and corresponding increase in the fraction of receptors exhibiting the lower apparent affinity. This change in agonist binding properties is not brought about by exposure of cells to antagonists. Exposure of cells to partial agonists results in conversion of a smaller fraction of the receptors to the altered form. The reaction occurs rapidly and is fully reversible. The $K_{0.5}$ for isoproterenol for this reaction is similar to that for stimulation of cyclic AMP accumulation in these cells (Fig. 6) and to that for induction of receptor-specific desensitization (19).

The ability to detect a form of the receptor exhibiting very low apparent affinity in short time assays, both in control

cells and in cells preincubated with agonists, does not depend simply upon whether the competing ligand used is an agonist or an antagonist, since these phenomena occur for the antagonist sotalol as well as for the agonists isoproterenol and epinehrine (Figs. 1–4 and Table I). In contrast, the antagonists metoprolol (Fig. 3) and propranolol (data not shown) exhibited single site, high affinity competition in both control cells and in cells preincubated with agonist. These agonists and sotalol are more hydrophilic ligands than metoprolol and propranolol (21, 22), and we propose that it is this property of these ligands that is responsible for the low apparent affinity observed in short time assays. The very low apparent affinity for hydrophilic ligands of the altered form of the receptor observed in 15-s assays with cells pre-exposed to agonist most likely results from slow equilibration of isoproterenol with this population of receptors (Fig. 2). We discuss below a model that can explain both the low apparent affinity for hydrophilic ligands in short time assays and the apparent slow equilibration of these ligands with the altered form of the receptor.

A small fraction of the receptors exhibited low apparent affinity for agonists even in control cells (Fig. 1). This could result from rapid formation of the altered form of the receptor even during the short assays used here, or alternatively it could indicate that even in the native state a portion of the receptors exist in a form with properties similar to that formed upon incubation with agonists. The results presented for sotalol competition in Fig. 3*B* argue strongly in favor of the latter explanation. Sotalol does not induce formation of the altered form of the receptor, yet a similar fraction of the receptors in control cells exhibit low apparent affinity for sotalol in short time assays as for isoproterenol. In addition, if rapid formation during the assay were to account for these low apparent affinity receptors, the process for their formation would have to occur much more rapidly than indicated by the time course shown in Fig. 5. These receptors in control cells exhibit affinities for both isoproterenol and sotalol in short time assays that are similar to those of the altered receptors formed upon preincubation with agonists. In addition, sotalol apparently exhibits the same phenomenon of slow equilibration with this population of receptors in naive cells as isoproterenol does with the altered receptors formed during incubation with agonists. Taken together, these results suggest that in control cells a portion of the β-adrenergic receptors exist in a form of low apparent agonist affinity that may be the same as that formed upon incubation in the presence of agonists.

Results obtained in our studies with the C62B cell line (Fig. 7) were completely analogous to those with 1321N1 cells, and the results obtained by Pittman and Molinoff (8) with L6 rat muscle cells suggested that a similar reaction might occur in this cell line. However, they did not show full competition curves and so it is not possible to tell whether a portion of the receptors in control L6 cells also exhibit low apparent affinity for agonists, nor is it possible from their results to quantitatively determine the affinity of the receptors in these cells in the native state. Preliminary reports (18, 23)[4] indicate that a similar reaction also occurs in S49 mouse lymphoma cells. Taken together, these results suggest that a reaction similar to that described here may be a general feature of β-adrenergic receptor modulation by agonists.

To determine the possible relationship of the changes in receptor binding properties reported here to agonist-induced desensitization, we studied agonist-induced cyclic AMP accumulation under short time assay conditions similar to those used to study binding to intact cell receptors. In 1321N1 cells, preincubation with isoproterenol for 20 min led to a concentration-dependent decrease in the maximal cyclic AMP response, along with a 2–3-fold increase in the value of K_{act}. The decrease in maximal response correlated reasonably well with the fraction of receptors converted from the high affinity form to the altered form. These results suggest that in these cells the full complement of high affinity receptors is required to maintain maximal hormone responsiveness.

The results obtained with the C62B cells were somewhat different. Preincubation for 20 min with a saturating concentration (1 μM) of isoproterenol did not lead to any decrease in the maximal response, but rather to a small increase in maximal response together with a 3-fold increase in K_{act}. This is in spite of a 30% decrease in the total number of assayable receptors and a shift of approximately half of the remaining receptors from the high affinity form to the altered form. These cells can thus maintain the same level of maximum responsiveness to isoproterenol in spite of a 3-fold decrease in the number of high affinity receptors. This result is consistent with previous studies (24, 25) which suggest that the C62B cell line may have a high efficiency of coupling of β-adrenergic receptors to adenylate cyclase and/or some degree of "spareness" of receptors.

Several characteristics of the altered form of the receptor produced upon incubation with agonists lead us to suggest that it is most likely present inside the cell within an endocytotic vesicle. Hydrophilic ligands, such as isoproterenol, epinephrine, and sotalol, would be expected to diffuse across the plasma membrane of intact cells only slowly. Thus, in short time assays, the concentration of these hydrophilic ligands inside the cell would be much lower than the concentration outside the cell and little inhibition of radioligand binding to internalized receptors would be seen at concentrations of competing ligand that would effectively block binding to cell surface receptors. However, at very high concentrations of hydrophilic ligand, sufficient permeation of competing ligand into the cell would occur to inhibit radioligand binding to internalized receptors as well, even in very short assays. With increasing time of exposure to varying concentrations of competing hydrophilic ligand, further permeation of ligand into the intracellular compartment containing the internalized receptor would occur, and the concentration of competitor required outside the cell to achieve an effective inhibitory concentration inside the cell would decrease, thus leading to a leftward shift in the observed competition curve. The idea of internalized receptors could thus explain both the low apparent affinity observed in short time assays as well as the apparent slow equilibration observed with hydrophilic ligands. The slow equilibration of these ligands would then not be due to a slow rate of interaction of the ligand with the receptor but rather an artifact due to slow permeation of the ligand into the cellular compartment containing these receptors. The more lipophilic ligands metoprolol and propranolol might be expected to freely diffuse through the plasma membrane, and thus even at short assay times these ligands would appear to bind with equal affinity to both cell surface and internalized receptors; thus, a single class of binding sites would be observed, both in control cells and in desensitized cells.

Previously reported studies from this laboratory also are

[4] *Note Added in Proof*—After submission of this manuscript a publication appeared on the same subject (Insel *et al.* (1983) *J. Biol. Chem.* **258**, 13597–13605). The results of Insel *et al.* are complementary to our previous results (1) and to the results presented in this article. They extend the basic observation of an agonist-induced, time-dependent formation of an altered form of the β-adrenergic receptor to the S49 mouse lymphoma cell line.

consistent with the idea of agonist-induced internalization of β-adrenergic receptors (26). Sucrose density gradient centrifugation studies of β-adrenergic receptors in membrane preparations from both control 1321N1 cells and cells preincubated with agonists were performed. In control cells, the bulk of the receptors migrated in a heavy density fraction together with markers for the plasma membrane, with a small fraction of the receptors migrating in a light vesicle fraction. In desensitized cells, there was an apparent shift of about half of the receptors from the heavy fractions to the light fractions. These light peak receptors were interpreted as evidence for agonist-induced internalization of receptors within endocytotic vesicles. Similar results also were obtained with C62B cells (27).

We propose that the receptors exhibiting low apparent affinity for agonists in control cells in the present study are those migrating in the light peak in membranes prepared from these cells, and that the increase in this population of receptors upon desensitization corresponds with the increase in light peak receptors in membranes from desensitized cells. In both control and desensitized cells, the proportions of total cellular receptors in the high and low apparent affinity forms correspond to the proportions of receptors in the heavy and light peak fractions, respectively, in membrane preparations (26). The time courses for agonist-induced formation of light peak receptors (28) and for formation of the altered form of the receptor as measured in the present study (Fig. 5) are very similar, including in both cases the presence of a 30–45 s lag before any altered receptors can be detected. The agonist concentration dependence of these two phenomena and the time courses for their reversal are also similar (Fig. 5 and Refs. 26 and 28). In addition, pretreatment of cells with concanavalin A prior to incubation in the presence of agonist prevents both the alteration in receptor binding properties described here[5] and the formation of light peak receptors (28), but does not block the agonist-induced uncoupling of β-adrenergic receptors from adenylate cyclase. If in fact the altered receptors are intracellular as we postulate, then those present in control cells might be an indication of continuous recycling of receptors between intracellular and cell surface compartments even in the absence of agonists or might represent newly synthesized receptors not yet transported to the cell surface.

Recently reported studies of β-adrenergic receptors on intact C62B cells using the radioligand ^{3}H-CGP-12177 also are consistent with the idea of receptor internalization (29–31). This radioligand is relatively hydrophilic and appears to label only cell surface receptors (29, 30). In control cells, isoproterenol exhibits high affinity competition for ^{3}H-CGP-12177 binding sites even in equilibrium binding assays (30). This result would be expected if the altered receptors are in fact internalized and thus unavailable for binding of this hydrophilic radioligand. Preincubation of cells with isoproterenol leads to an approximately 50% decrease in the number of receptors available for binding of this ligand (29), consistent with internalization of half of the receptors. Whether the remaining cell surface binding sites for this ligand retain high affinity for agonists was not presented, although the results obtained in the equilibrium competition binding assays (30) would suggest that this would be the case. Furthermore, when sucrose density gradient centrifugation was performed on membranes from C62B cells labeled with ^{3}H-CGP-12177 prior to lysis, only the heavy peak receptors were found to be labeled (31). Thus, the receptors with high affinity for agonist detected with this ligand in intact cells are apparently the plasma membrane receptors (which migrate in the heavy peak) and the receptors that are inaccessible to this ligand in intact cells are those that migrate in the light peak.

Recent studies with frog erythrocytes suggest that receptor internalization may also be involved in desensitization of these cells (32–35). Early studies reported that desensitization of these cells was due to loss of β-adrenergic receptors (32). More recent studies (34) have shown that in fact these "lost" receptors can be recovered, apparently in a light vesicle fraction, by high speed centrifugation. These receptors are most likely analogous to the light peak receptors identified in both 1321N1 and C62B cells. Further studies of the light vesicle receptors from frog erythrocytes led Strulovici *et al.* (35) to conclude that the receptors in these vesicles were fully functional, and that the functional desensitization of these cells was due to sequestration of the receptors away from the guanine nucleotide binding protein and adenylate cyclase.

High and low agonist affinity states of the β-adrenergic receptor have previously been demonstrated in membrane preparations from a variety of sources, including 1321N1 cells (19, 36, 37). The high affinity state can be demonstrated only in the absence of guanine nucleotides and is believed to represent a ternary complex between agonist, receptor, and the guanine nucleotide binding protein (37, 38). Addition of guanine nucleotides to the assays dissociates the guanine nucleotide binding protein from this complex and converts all of the receptors to the low affinity state. In membrane preparations, desensitized receptors exhibit only low affinity binding even in the absence of guanine nucleotides (26, 34, 40). This result has been interpreted as an indication that desensitization causes an "uncoupling" of the β-adrenergic receptors from the guanine nucleotide binding protein. It would seem likely that in the intact cell there would be sufficient guanine nucleotide to prevent accumulation of β-adrenergic receptors in the ternary complex high affinity form. We therefore think it likely that the state of the receptor observed in intact cell assays as high affinity most likely reflects the lower affinity state observed in control membrane in the presence of guanine nucleotides.

The K_D for isoproterenol binding to β-adrenergic receptors in membrane preparations from desensitized 1321N1 cells in the presence of guanine nucleotides is in the range of 0.2–0.6 μM (26), whereas the equilibrium K_D for isoproterenol binding to the low affinity receptors in intact cells is in the range of 8–12 μM (Fig. 2 and Ref. 1). Thus, the state of the receptor observed as the low affinity form in equilibrium assays in intact desensitized cells is apparently not detected in membrane preparations from these cells. This could indicate that the modification of the receptor that is responsible for the low equilibrium affinity in intact cells is unstable to the conditions used to lyse cells and prepare membrane fractions. Alternatively, the apparent low affinity observed at equilibrium in intact cells could be an artifact due to intracellular metabolism of agonist or to differential partitioning of agonist into the extracellular, intracellular, and intravesicular compartments, and thus might not represent any change at all in the molecular properties of the receptor, as suggested by Strulovici *et al.* (35). In addition, we should point out that due to the variety of rapid, agonist concentration-dependent changes in receptor number, distribution, and ligand properties that can occur in intact cells during the course of an equilibrium competition binding assay with agonists, it may not be possible to ever truly achieve equilibrium or even a true steady state of binding in intact cell assays. Thus, it may be inappropriate to interpret the apparent affinity observed in such assays as being due to simple competition by the

[5] M. L. Toews, G. L. Waldo, T. K. Harden, and J. P. Perkins, manuscript in preparation.

agonist for binding to the intact cell receptor. In fact, because of the above consideration, short time assays such as those used in our studies may be much more appropriate than conventional equilibrium assays for investigating the agonist binding properties of β-adrenergic receptors on intact cells.

To summarize, the studies reported here describe an agonist-induced change in the agonist binding properties of β-adrenergic receptors on intact cells that seems clearly related to the overall process of agonist-induced receptor-specific desensitization. The properties of the altered form of the receptor are entirely consistent with the hypothesis that it results from internalization of a portion of the cell surface receptors. The nature and significance of the apparent low affinity for agonists observed in equilibrium assays with intact cells and the relationship to this phenomenon of the agonist-induced changes reported here remains unknown.

Acknowledgments—We wish to thank Dr. T. K. Harden for numerous helpful discussions throughout the course of this work, Drs. R. N. Pittman and P. B. Molinoff for sharing unpublished results, and Sherry E. Jones for her expert technical assistance.

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